The Membrane Proteins of the Vacuolar System II. Bidirectional Flow between Secondary Lysosomes and Plasma Membrane

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ABSTRACT Lactoperoxidase covalently coupled to latex spheres (LPO-latex) has been used to selectively iodinate the phagolysosome (PL) membrane within living macrophages, as discussed in the accompanying article. This procedure labeled ~24 polypeptides in the PL membrane; these were similar to those iodinatable on the external surface of the plasma membrane (PM). We now report on the translocation and fate of these proteins when the cells are returned to culture.

TCA-precipitable radioactivity was lost from cells with biphasic kinetics. 20–50% of the cell-associated radiolabel was rapidly digested ($t_{1/2} \cong 1$ h) and recovered in the culture medium as monoiodotyrosine. 50–80% of the label was lost slowly from cells ($t_{1/2} \cong 24$–30 h). Quantitative analysis of gel autoradiograms showed that all radiolabeled proteins were lost at the same rate in both the rapid and slow phases of digestion.

Within 15–30 min after labeling of the PL membrane, EM autoradiography revealed that the majority of the cell-associated grains, which at time 0 were associated with PL, were now randomly dispersed over the plasmalemma. At this time, analysis of PM captured by a second phagocytic load revealed the presence of all labeled species originally present in the PL membrane. This demonstrated the rapid, synchronous centrifugal flow of PL polypeptides to the cell surface.

Evidence was also obtained for the continuous influx of representative samples of the PM into the PL compartment by way of pinocytic vesicles. This was based on the constant flow of fluid phase markers into latex-containing PL and on the internalization of all iodinatable PM polypeptides into this locus.

These observations provide evidence for the continuous, bidirectional flow of membrane polypeptides between the PM and the secondary lysosome and represent an example of a membrane flow and recycling mechanism.

In the preceding paper we described a technique for selectively iodinating the luminal surface of the phagolysosomal (PL) membrane by interiorizing lactoperoxidase conjugated to latex spheres. Radiolabel was recovered as monoiodotyrosine and was present in at least 24 separate polypeptides (12,000–250,000 daltons) when displayed on one-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels. These labeled proteins were essentially identical to those labeled in the plasma membrane by surface iodination (12).

In this report we use these methods to gain further insight into the dynamics of membrane flow in the vacuolar apparatus. We demonstrate that radiolabel that is initially in the PL membrane moves rapidly to the cell surface and is randomly associated with the plasma membrane. Isolation of plasma membrane after the redistribution of label indicates that all the polypeptides initially iodinated within the PL take part in this flow. Simultaneously, plasma membrane with its full complement of polypeptides is being interiorized as pinocytic vesicles and is entering the PL compartment. These studies demonstrate in a direct fashion the continuous and bidirectional flow of

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membrane polypeptides from the vacuolar apparatus to the cell surface.

MATERIALS AND METHODS

Materials

Styrene-butadiene latex spheres (S-B latex), 0.527 μm in diameter, were purchased from Dow Diagnostics, Inc., Indianapolis, Ind. Chymotrypsin was purchased from Worthington Biochemical Corp., Freehold, N. J. All other reagents were as described in the preceding paper.

Fate of the Incorporated Radiolabel

Most techniques used in this work, except as noted below, have already been described in the preceding paper (12).

After ingestion of lactoperoxidase (LPO)-latex, macrophage monolayer cultures were iodinated intracellularly, washed in ice-cold phosphate-buffered solution (PBS) containing KI and then in PBS, and returned to the incubator in fresh medium at 37°C. Upon removal from culture, they were always washed at least three times with PBS. The fate of the radiolabel was followed in the following ways:

(*a*) TCA precipitation was performed on lysed cell monolayers.

(*b*) SDS-PAGE was performed on cell lysates and on subcellular fractions from discontinuous sucrose gradients.

(*c*) EM autoradiography (EM-ARG) was performed on thin sections of fixed, embedded cells and analyzed as described previously (12).

(*d*) Protease treatment was conducted after iodination. At various times after return to culture, cells were washed in PBS and incubated for 5 min at 37°C in PBS or PBS containing 100 μg/ml chymotrypsin. (These solutions had been warmed to 37°C before being added to the cells.) The supernates were collected, passed through a Millex filter (Millipore Corp., Bedford, Mass.) to remove any detached cells (≪ 1% of the total number), and lyophilized. The freeze-dried material was redissolved in 1 M acetic acid and chromatographed on Sephadex G-25. The cell monolayers were lysed in 0.05% Triton X-100 and precipitated with 10% TCA containing 100 mM KI.

Isolation of Plasma Membrane

The following method was devised to retrieve representative samples of radiolabeled plasma membrane (PM) polypeptides at any given time. Its success depends upon the density differences between LPO-latex PL and S-B latex PL and upon the use of a large dose of S-B latex to incorporate a large area of PM into phagocytic vesicles within a short time. Immediately after intracellular iodination with LPO-latex beads or 30 min after return to culture, cells were washed and a 1:50 dilution of the 10% stock of low density (ρ = 1.027) S-B latex particles was centrifuged down upon the monolayer at 1,000 *g* for 5 min at 4°C. The cultures were washed with PBS and warmed to 37°C for 5 min to allow ingestion of the S-B latex. Each cell ingested several hundred beads during this time, compared to the several dozen LPO-latex beads already present within the cells.

The cells were then scraped from the plates and homogenized in isotonic sucrose. 2.5 ml of the homogenate was brought to 35% sucrose (wt/wt) and overlaid first with 6 ml of 15% sucrose and then 2.5 ml of 5% sucrose. All sucrose solutions contained the protease inhibitors phenylmethyl sulfonyl fluoride (PMSF) and aprotinin. The discontinuous gradient was centrifuged at 100,000 *g* for 1 h at 4°C. Fractions were collected and processed for TCA precipitation, latex quantitation, and SDS-PAGE. LPO activity was assayed by triiodide production.

Microscopic examination (× 1,000) and enzymatic assay of the fractions showed that the 5/15% interface contained about two-thirds of the total S-B latex but no detectable LPO-latex. The 15/35% interface contained most of the LPO-latex and a few S-B latex. The pellet fraction also contained both latex types. Therefore, the material in the 5/15% interface represented plasma membrane as it existed at the time of S-B latex addition as well as membrane that joined the plasmalemma or S-B latex phagosome during the 5-min ingestion period.

Fusion of Pinocytic Vesicles with LPO-latex PL

Three approaches were used to document fusion of pinocytic vesicles with PL:

ACCUMULATION OF A PARTICULATE MARKER: Macrophage monolayers were allowed to ingest LPO-latex as usual, washed, and returned to culture for 9 h in fresh medium containing 0.5% colloidal thorium dioxide (Thorotrast). Cells were washed, fixed, exposed to 3,3'-diaminobenzidine tetrahydrochloride (DAB)-H_2O_2 to visualize LPO (8) and further processed for transmission EM to examine the Thorotrast accumulation in LPO-latex PL.

ACCUMULATION OF FLUID MARKER: Macrophage monolayer cultures were allowed to ingest a dose of unmodified polystyrene latex spheres (PS-latex) equivalent to the usual LPO-latex load. The cells were then returned to culture with 1 mg/ml horseradish peroxidase (HRP) in the medium. After various times of incubation, the cells were washed extensively and returned to culture for 20 min in medium without HRP. The cells were then homogenized and fractionated on a 10/25/35% sucrose gradient. Peroxidase enzymatic activities of the whole-cell homogenate and the purified PL fraction (10/25% interface) were determined by the *o*-dianisidine assay. Latex was quantitated by light scattering and protein by the method of Lowry et al. (11).

ACCUMULATION OF RADIOLABELED PM: Macrophage monolayers were allowed to ingest unmodified PS-latex as above. The cells were then chilled, and their external surfaces were iodinated on ice for 10 min, using soluble LPO (40 mU/ml) and glucose oxidase (GO) (10 mU/ml). The cells were washed and either homogenized immediately or returned to culture for 30 min before homogenization. The homogenate was fractionated on a 10/25/35% sucrose gradient.

RESULTS

Fate of Radiolabeled PL Membrane Polypeptides

Initial studies were focused on the turnover of the iodinated polypeptides of the PL membrane. Phagocytosed LPO-latex was used to iodinate the PL membranes of mouse macrophages and the cells were returned to culture. The macrophages remained viable for at least 1 wk, and their morphologic features were indistinguishable from cells receiving a phagocytic load of unmodified latex. Quantitation of cell-associated latex by

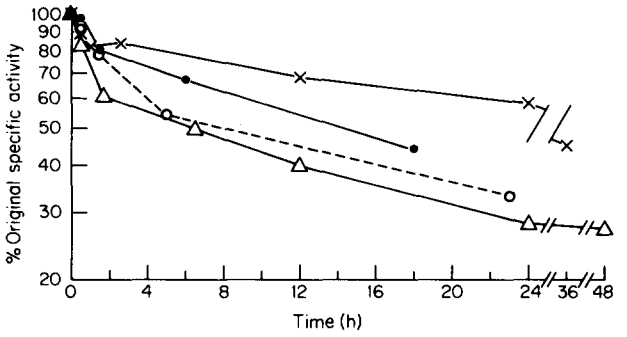


FIGURE 1 Kinetics of radiolabel loss from internally iodinated macrophages. After intracellular iodination, cells were returned to culture for the times indicated, then washed, lysed, and assayed for TCA-precipitable radioactivity and protein. The curves are from four separate experiments performed over the course of 1 yr. Each point represents the mean of triplicate measurements. The standard deviation was always within 10%. (The original specific activities [cpm/μg of cell protein] were: × = 1,662, ○ = 37,352, ● = 758, and △ = 584.)

TABLE I

Identification of Radiolabeled Species in Cells and Culture Medium

Sample	Time in culture	Radioactivity	
		Void volume	MIT
	h		
Cells	0	15,363	0*
Cells	2	5,882	238
Medium	2	0	10,042

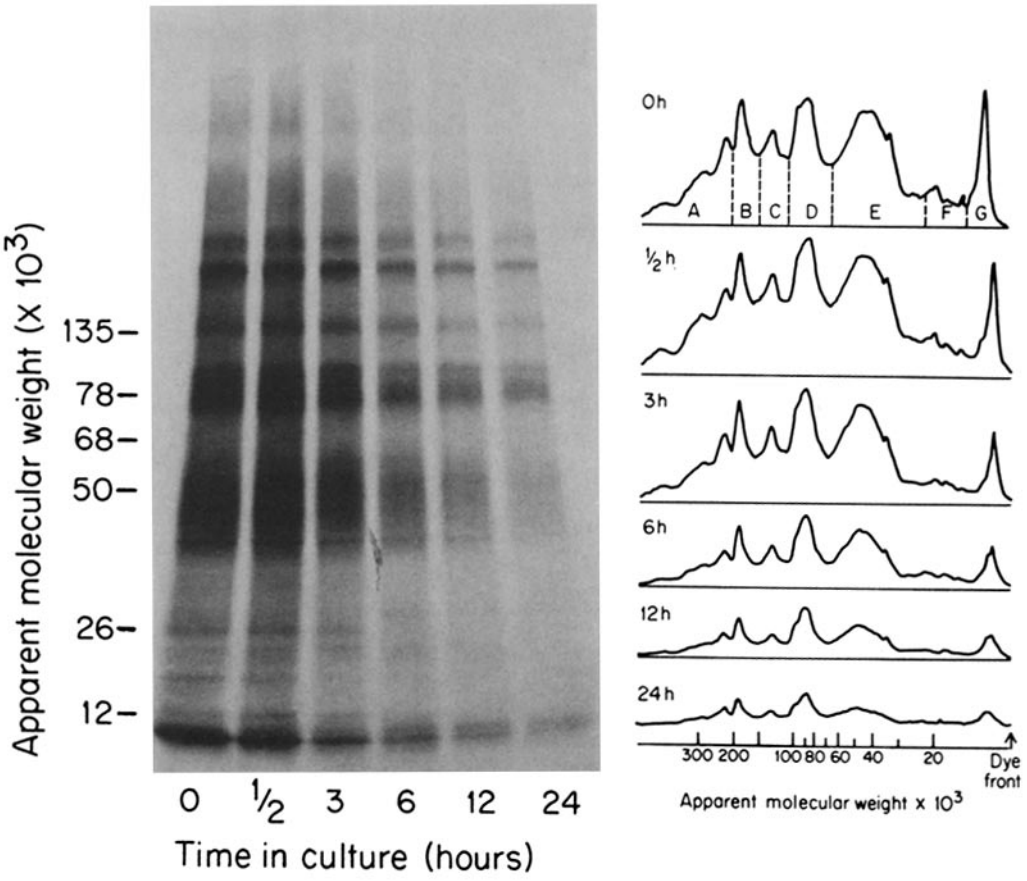
After intracellular iodination or 2 h after return of cells to culture, the medium was aspirated and cells scraped from the dish with a plastic policeman. The medium and cells were lyophilized immediately and the concentrated material was chromatographed on Sephadex G-25 (12).

* Not detectable above background.

direct microscopic examination at high power, and by light scattering of cell lysates, showed that all of the LPO-latex originally ingested remained within the cells. LPO activity of the ingested latex remained stable for ~5 h (triiodide enzymatic assay) but fell to one-tenth the original activity by 24 h.

The TCA-precipitable radiolabel that had been incorporated into PL was lost from the monolayer with biphasic kinetics (Fig. 1). Identical kinetics were obtained whether or not trypsinization was employed to remove extracellular beads. There was first a rapid loss ($t_{1/2} \cong 1$ h) of 20–50% of cell-associated radioactivity. The size and rate of this rapid component was unaffected by the amount of radiolabel incorporated within the range we have tested (Fig. 1). This early rapid loss of TCA-precipitable counts was the result of extensive digestion of radiolabeled proteins to the amino acid level. Lost TCA-precipitable counts were recovered quantitatively as radiolabeled monoiodotyrosine ($M^{125}IT$) when the culture medium was chromatographed on Sephadex G-25 to separate MIT from the free iodide that also eluted (Table I).

The bulk of the label (50–80%) was degraded slowly, with a $t_{1/2}$ of 24–30 h or longer (Fig. 1). SDS-PAGE was then employed to characterize cell-associated radiolabel at various times after return to culture (Fig. 2). Although the total radioactivity decreased with time, the same iodinated species were observed at all times. In addition, quantitative densitometric analysis of the autoradiogram showed that the relative intensity of isotope among the bands was preserved (Fig. 2). This indicated that all of the labeled polypeptides were subject to



Hours after return to culture	Region						
	A	B	C	D	E	F	G
	% of total area						
0	16.5	9.9	9.7	17.5	27.9	6.5	7.7
0.5	16.4	8.3	10.2	17.9	29.1	5.9	7.1
3	16.9	9.6	9.1	18.3	31.1	5.0	5.9
6	16.1	9.5	8.8	18.1	29.5	7.6	7.2
12	16.2	9.6	6.8	19.8	34.6		8.9
24	16.5	9.6	6.5	18.6	25.7	5.7	8.3

FIGURE 2 Turnover of labeled phagolysosomes polypeptides. After intralysosomal iodination, macrophages were returned to culture. At the times indicated, cultures were lysed and samples containing equal amounts of protein were subjected to SDS-PAGE (4–11% gradient gel). Top left, autoradiogram of dried gel (4-d exposures on Kodak XR-1 film with enhancing screen). Molecular weights of gel standards are indicated at the left. Top right, densitometer tracings of the autoradiogram. The curve has been broken up into peak regions (indicated by the vertical lines) by an integrating circuit attached to the densitometer that also calculated the percent of total area under each peak. These values are tabulated.

both rapid and slow phases of degradation and turned over at a similar rate.

The presence of a large pool of free ^{125}I immediately after iodination raised the possibility that radiolabel might continue to be incorporated and generate an artificial slow phase. We therefore exposed LPO-latex-containing cells to the standard iodination conditions in the presence or absence of 6 mM (0.02%) sodium azide, which reversibly inhibits LPO activity. Although azide completely inhibited intracellular iodination (Table II and the preceding paper), a pool of free ^{125}I accumulated nevertheless. When these cells were washed and returned to culture, they failed to incorporate any radiolabel into TCA-precipitable material over a 2-h period (Table II). As a control, we incubated azide-treated cells with fresh ^{125}I and GO. The ^{125}I was incorporated, proving that the LPO-latex was still enzymatically active. We conclude that after the initial intracellular iodination at 4°C in our system, macrophages did not incorporate additional ^{125}I after return to culture.

Redistribution of Radiolabel from the PL Membrane—EM-ARG

The preceding studies indicated that the majority of the polypeptides initially iodinated in the PL membrane remained intact and cell associated. We next employed EM-ARG to examine their distribution in cells incubated at 37°C. We had previously found that, immediately after intracellular iodination, exposed silver grains were located over LPO-latex PL (Fig. 3*a* and the preceding paper). When the cells were returned to culture for 30 min, a striking redistribution of grains was observed (Fig. 3). Now, large numbers of grains were present on the cell surface and found over the PM. Some grains also lay over the cytoplasm, particularly in vesicle-rich areas, and some labeling of LPO-latex PL was still evident (Fig. 3). The redistribution of label to the PM was readily detected within 15 min of culture (the earliest time-point studied). In addition, the distribution of grains in EM-ARG performed .5, 1, and 24 h after return to culture was similar and suggested that a steady state had been reached at the earliest time-point.

For quantitative analysis, autoradiograms were prepared by the flat substrate method and analyzed by the probability circle method as described in the previous paper. Analysis of 530 grains in 48 randomly selected cell profiles showed that 78% of the grains were located over PM at 30 min (Table III). The density of grains on the dish and upper surfaces of the PM were equal. Relatively few grains were associated with LPO-latex at this time, although the grain density of this compartment approached that of the PM, when counts were corrected for the relative areas of PM and PL membrane (Table III). Compartments other than PM and PL contained little label.

We conclude that by 30 min after return to culture the radiolabel had redistributed from the PL to the PM.

Attempts were made to quantitate the rate of radiolabel appearance on the cell surface by means of short periods of proteolysis (100 μg/ml chymotrypsin for 5 min at 37°C) at various times after return to culture. Such a short period was necessary because redistribution of label was so rapid and because chymotrypsin could enter the cell by pinocytosis. The material released into the extracellular medium was concentrated and analyzed by Sephadex G-25 chromatography. Immediately after iodination, cell-associated radiolabel was completely resistant to extracellular protease (Table IV). Within 30 min of additional culture, however, maximal quantities of protease-sensitive radioactivity were observed. The percent of total cell radiolabel susceptible to proteolysis was small in absolute terms (~2.5% of the total), but corresponded to half the level that could be removed by a similar treatment of macrophages iodinated externally (Table IV). We conclude that some of the radiolabel redistributing from PL to PM is protease sensitive; however, the low levels of radioactivity released under these conditions prevented further use of this approach.

TABLE II

Residual $^{125}I^-$ Is Not Incorporated upon Return to Culture

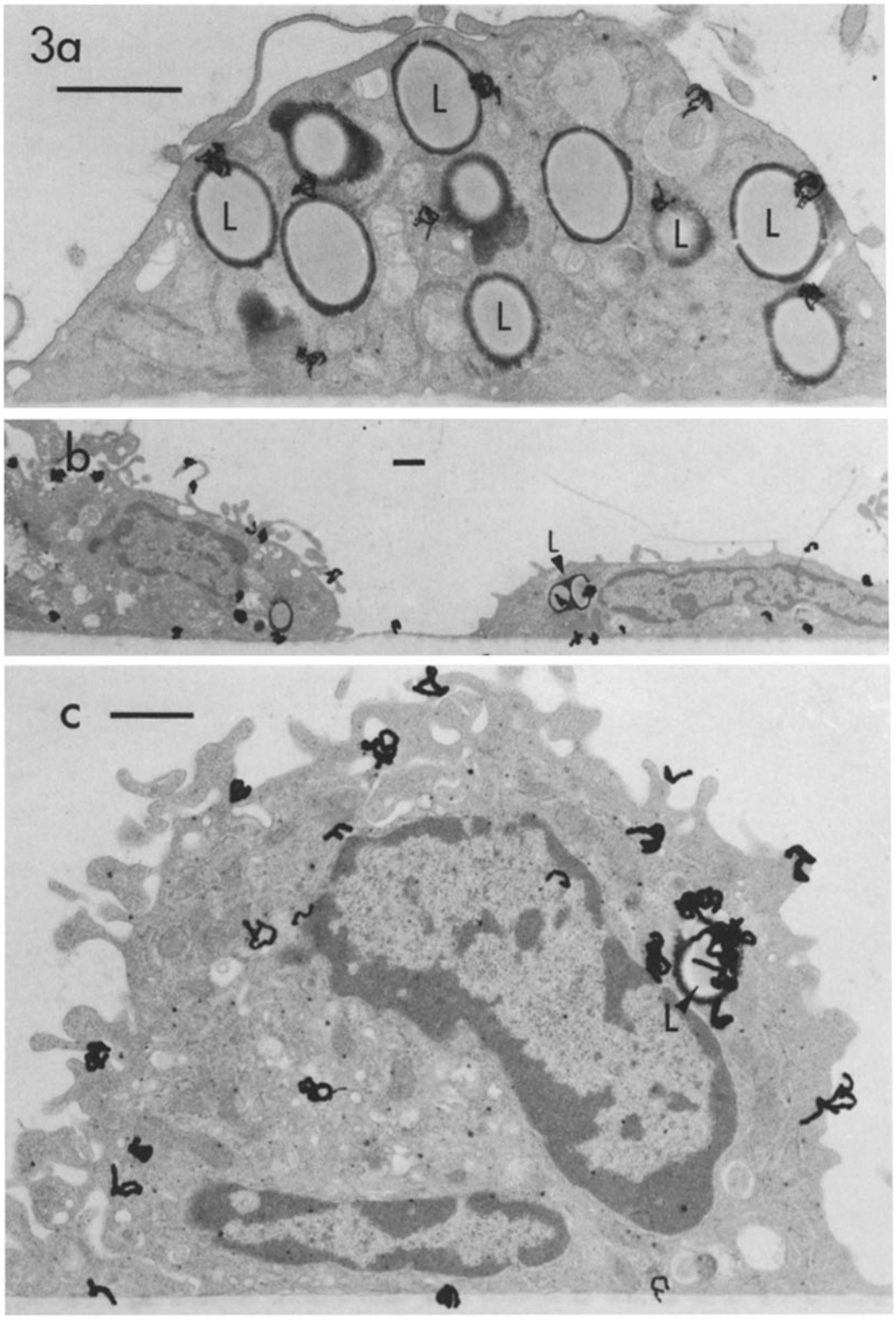
Azide during iodination	Return to culture in	Time in culture	Specific radioactivity
		h	*cpm/μg of cell protein*
−		0	1,569 ± 238
+		0	52 ± 16
+	Culture medium	0.5	39 ± 10
+		2.0	50 ± 23
+	$^{125}I^-$, glucose, GO	0.5	1,687 ± 444
+		2.0	1,753 ± 235

2×10^6 resident peritoneal cells were plated in 16-mm Costar wells and cultured for 2 d in medium 199 supplemented with 10% fetal calf serum. After ingestion of 1:800 LPO-latex, they were exposed to iodinating conditions (40 μCi/ml $^{125}I^-$, 0.24 mU/ml GO) on ice in the presence or absence of 6 mM (0.02%) NaN_3. The latter treatment blocks iodination but allows the cells to accumulate $^{125}I^-$.

After 30 min, some of the azide-treated cultures were washed and returned to the 37°C incubation for .5 or 2 h in either culture medium or fresh iodinating reagents.

Isolation and Identification of the PL Polypeptides Appearing on the PM

Next, it was necessary to characterize the iodinated PL polypeptides that returned to the cell surface. We therefore isolated samples of PM from cells that had been iodinated intracellularly, either immediately after iodination or 30 min after return to culture. To accomplish this, we administered a second and larger phagocytic load of very low density S-B latex particles. Discontinuous sucrose density gradients could be used to retrieve some two-thirds of the S-B latex phagosomes from cell homogenates (5/15% interface) free from LPO-latex PL, which banded at the 15/35% interface (see Materials and Methods). SDS-PAGE gels were then obtained, using equal portions of the 5/15% interface from cells homogenized 5 and 35 min after intracellular iodination. It should be noted that 5 min represents the minimum time required for the ingestion of S-B latex and precludes recovery of PM immediately after intracellular iodination. At 5 min, relatively little radioactivity could be retrieved in S-B latex phagosomes (Fig. 4). In contrast, after 30 min in culture, larger amounts of all the radioactive polypeptides were present on the cell surface and were captured in S-B latex phagosomes. Quantitative densitometric analysis of this autoradiogram showed that the relative intensities of the major bands (see Fig. 2) of the 5/15% fraction were the same at 5 and 35 min, although four times as much radioactivity was recovered at 35 min. This movement of label to the cell surface during 30 min of culture is even more striking, considering that the total radioactivity in the cells decreased in that interval (compare the 15/35% fraction at 5 and 35 min). The spectrum


3a
L
L
L
L
L
b
L
c
L

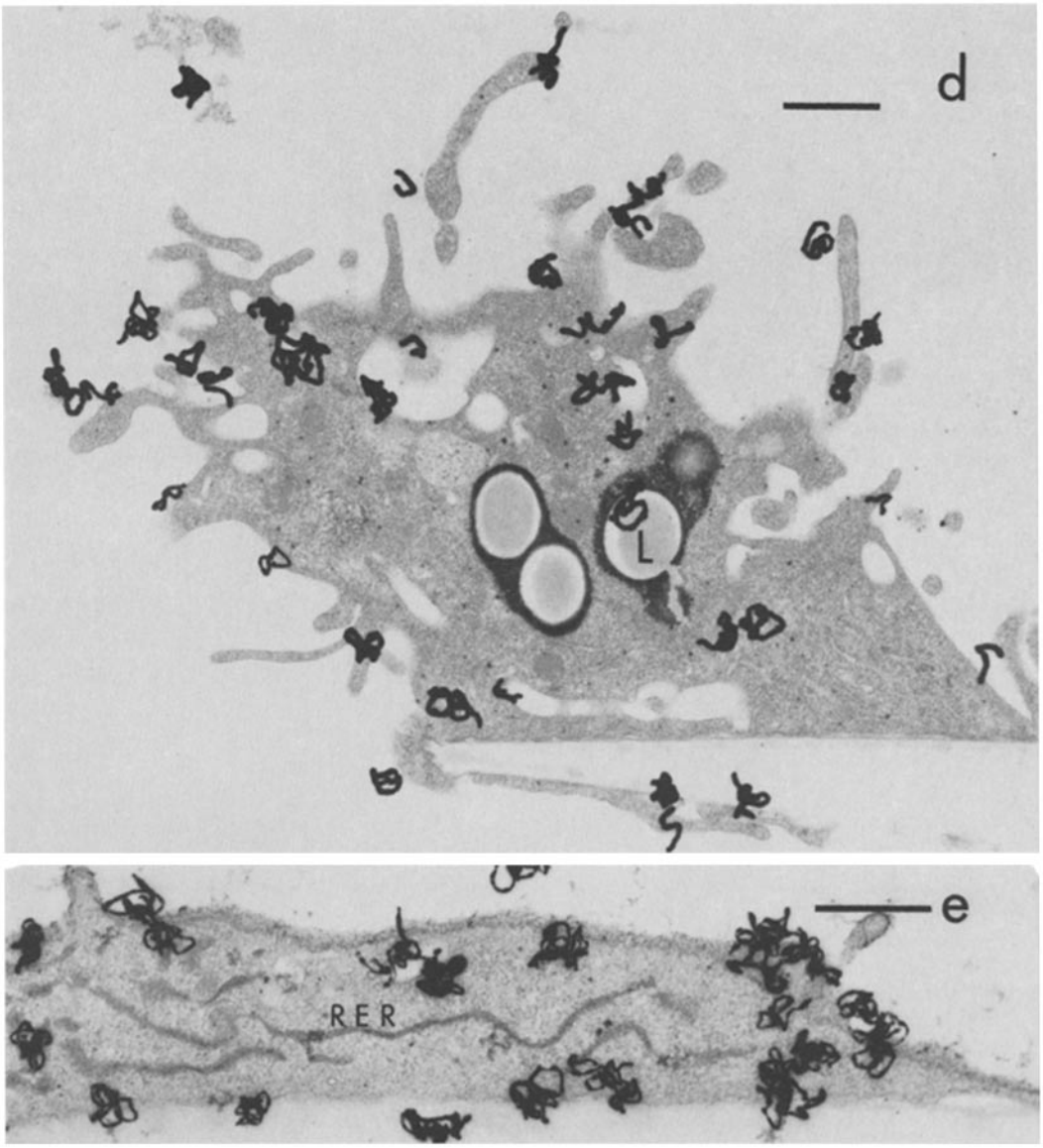


FIGURE 3 Redistribution of radiolabel from PL to plasma membrane. (*a*) Cell fixed immediately after iodination shows grains associated with LPO-latex PL (*L*). (*b–e*) Macrophages returned to culture for 30 min before fixation. Most of the grains are distributed over entire PM including the flat surface adherent to the culture dish. Some grains are still seen in association with LPO-latex. The cells shown in *b*, *c*, and *d* were stained with DAB-H_2O_2 for 10 min to mark LPO-latex. Ilford L4 emulsion, Microdol X developer, 2-wk exposure. The cells in *a* and *e* were from a different experiment. They were exposed to DAB-H_2O_2 for 30 min, a treatment that visualized peroxidase activity in rough endoplasmic reticulum (*RER*) and the perinuclear cisterna as well as LPO-latex PL. Ilford L4 emulsion; Kodak D-19 developer. 3.5-d exposure. Bars, 1 μm. (*a*) × 19,500, (*b*) × 5,000, (*c*) × 13,000, (*d*) × 13,000, (*e*) × 15,600.

of radiolabeled polypeptides in S-B latex PL was identical to that of the whole-cell homogenate (Fig. 4). We conclude that a representative sample of PL membrane proteins, iodinated intracellularly by LPO-latex, can return to the PM intact. Because PL membrane polypeptides are migrating in concert, the data suggest that intact segments of membrane are flowing from PL to PM.

The Influx of PM into the Latex PL

The movement of membrane from PL to PM without shrinkage of the phagolysosome implies that PL membrane must be replaced from other sources. Pinocytic vesicles (PV) fusing with PL would provide a logical new source of membrane (17). Evidence for this pathway was first obtained from the accumulation of extracellular pinocytic markers into PL. Transmission EM indicated that Thorotrast was delivered to all preexisting LPO-latex PL (Fig. 5). Quantitative enzymatic assays showed that HRP, a marker of fluid-phase pinocytosis, accumulated linearly in latex PL for at least 2 h (Fig. 6 *B*), paralleling its continuous uptake into cells (Fig. 6 *A*). About 1% of the total ingested HRP was recovered in the PL fraction, a value similar to those found for endogenous lysosomal hydrolases.

Evidence was next obtained that PV membrane (i.e., PM) itself became part of the PL. For this purpose we allowed macrophages to ingest unmodified latex and then iodinated the cell surface with soluble LPO at 4°C. The cells were homogenized immediately or 30 min after return to culture, and the PL fractions (10/25% interface) were analyzed by SDS-PAGE

TABLE III

Distribution of Grains 30 min after Return to Culture

	Plasma membrane	LPO-latex	Cytoplasm*	Nucleus	Mitochondria
Total grains (uncorrected)	383	25	89	33	0
Grains corrected for radiation spread from plasma membrane‡	383 (78.0%)	25 (5.1%)	61 (12.4%)	22 (4.5%)	0
Relative grain density§ A	1.91	0.86	0.42	0.21	
B	1.92	1.11	0.40	0.21	

* Cytoplasm includes ground cytosol, RER, Golgi complex, vacuoles, and all other structures not in separate categories. A grain was assigned to the cytoplasm compartment only when no other organelle fell within one half-radius from its center.

‡ Corrected for spillover of grains from the heavily labeled compartment as in reference 12.

§ Percent of total grains assigned to the compartment divided by the percent of total cell area occupied by that compartment. The data in row *A* were calculated based on the relative area of the entire LPO-latex bead profile. The data in row *B* were calculated using the relative area of the rim of the LPO-latex PL as the denominator.

TABLE IV

Protease Sensitivity of Cell Radiolabel

	Time in culture after iodination	Radiolabeled polypeptide released*
	h	
SURFACE IODINATION		
LPO-latex attached to surface of cell monolayers in the cold	0	4,709
INTRACELLULAR IODINATION		
LPO-latex phagocytosed by macrophages	0	0
	0.5	2,366
	5	2,694
	19	1,302

After iodination, cells were returned to culture for the times indicated, washed, and exposed to chymotrypsin (100 μg/ml) for 5 min at 37°C. The supernates were chromatographed on Sephadex G-25. See Materials and Methods for experimental details. Protein counts were not released at any time in the absence of chymotrypsin.

* Expressed as cpm removed per 10^5 cpm of cell radiolabel.

(Fig. 7). At time 0, little radiolabel was noted in the latex PL, but by 30 min over four times as much radiolabel (quantitated densitometrically) was recovered in the PL fraction (Fig. 7, *PL*). This PL radiolabel represented interiorized PM, because the iodination pattern was identical to that seen in the whole-cell lysate (Fig. 7, *H*). We conclude that PV can deliver representative samples of iodinated PM to latex PL and that PM flows rapidly into and out of the vacuolar system.

DISCUSSION

The data presented in this paper demonstrate directly that PM interiorized into PL can return intact to the cell surface. The experimental approach was to radioiodinate membrane proteins of the phagolysosome within living cells and then follow the fate of radiolabel when cells were returned to culture. Part of the radiolabeled protein was digested within the lysosome, as expected from previous studies (9, 19), but most of it returned to the plasmalemma.

Rapid Digestion of Radiolabeled PL Proteins

When macrophages were returned to culture after intracellular iodination of PL membrane proteins, there was a rapid loss of 20–50% of the TCA-precipitable radioactivity (Fig. 1). Radioactivity lost from cells was recovered as $M^{125}IT$ in the culture medium (Table I), indicating that extensive digestion of labeled proteins took place.

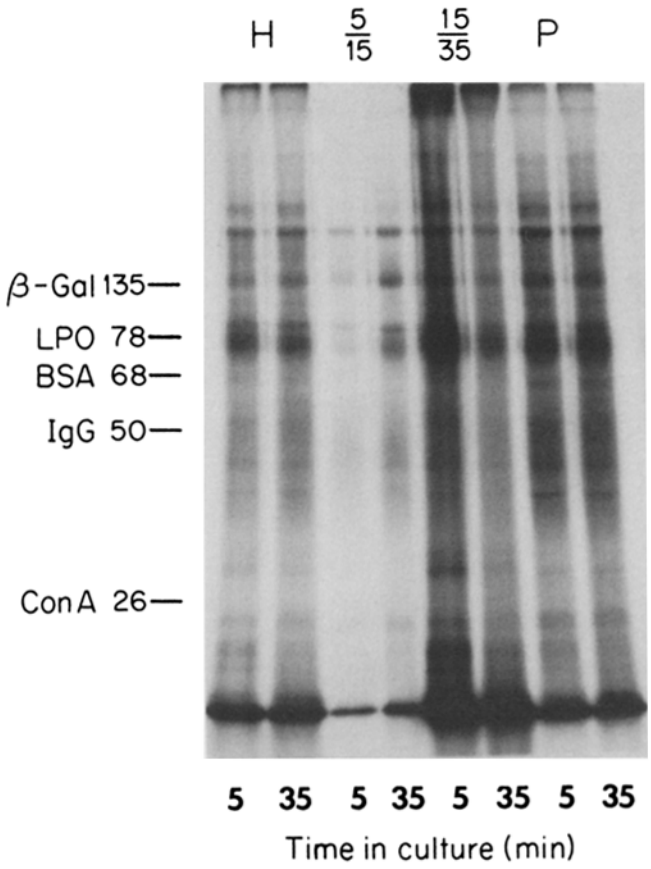


FIGURE 4 Identification of radiolabeled polypeptides in PM. Immediately after intralysosomal iodination (*5*) or after a 30-min return to culture (*35*), macrophages were given a 5-min phagocytic pulse of S-B latex, homogenized, and fractionated on a 5/15/35% discontinuous sucrose gradient. The fractions collected were analyzed by SDS-PAGE (4–11% slab gel). Samples of the total homogenate (*H*) and pellet (*P*) fractions contained equal TCA-precipitable radioactivity, whereas samples of the two interface fractions contained equal latex. The dried gel was exposed for autoradiography on Kodak XR-1 film with an enhancing screen. Molecular weights of gel standards (× 10^3) are at the left.

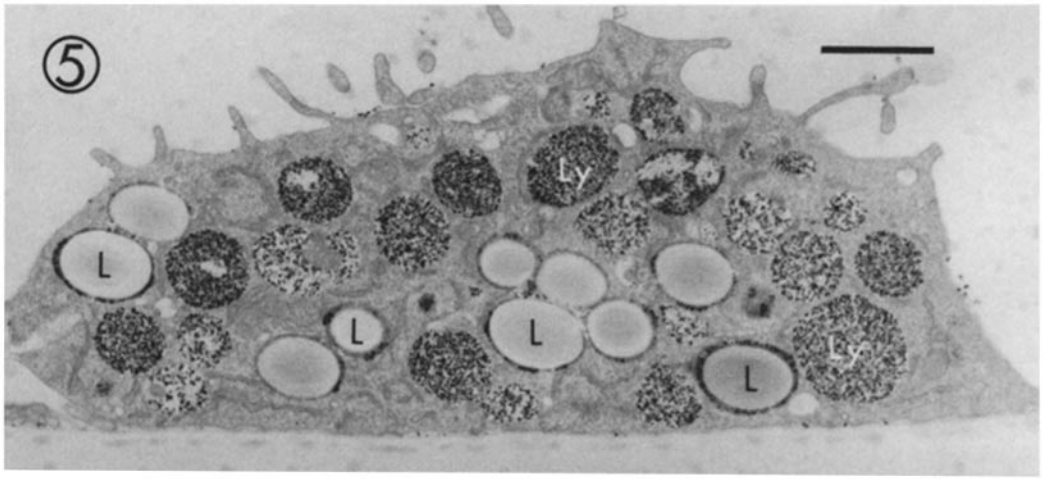


FIGURE 5 Accumulation of Thorotrast in cells previously fed LPO-latex. After ingestion of LPO-latex, cells were washed and returned to culture for 9 h in medium supplemented with Thorotrast. Note colloid particles within the latex phagolysosomes (*L*). Some enlarged lysosomes (*Ly*) are filled with colloid. The cells had been stained with DAB to mark LPO-latex with an electron-dense rim. Bar, 1 μm. × 16,000.

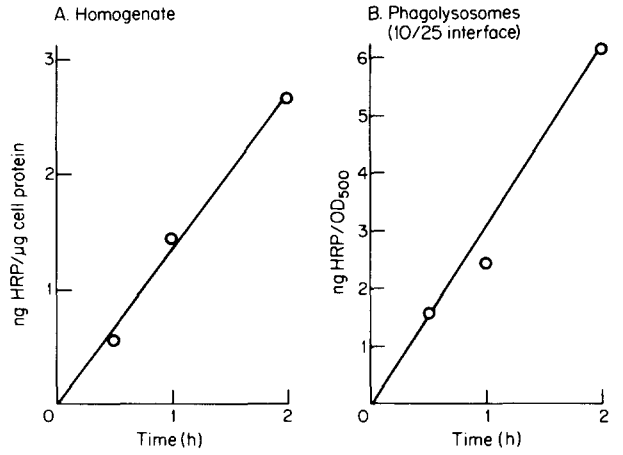


FIGURE 6 Uptake of HRP into latex PL. After ingestion of polystyrene latex particles, cells were exposed to HRP in culture medium for the times indicated, homogenized, and fractionated. (*A*) HRP activity in whole cell homogenate. (*B*) HRP activity recovered from purified PL, using OD_{500} to quantitate latex. Each point O represents the average of duplicate cultures fractionated in separate gradients. Placement of the lines was calculated by the method of least squares.

Lysosomal digestion of PM proteins interiorized by phagocytosis is a well-established phenomenon. Werb and Cohn (19) found that the PM enzyme 5′-nucleotidase was inactivated by macrophages after a latex meal, while Hubbard and Cohn (9) demonstrated that radioiodinated PM proteins of L cells were digested in lysosomes after latex ingestion. In these studies the percent of total PM marker digested could be increased by driving more membrane into the PL compartment with a larger phagocytic load.

We show that the radiolabeled PL membrane proteins of macrophages maintain the same intensities relative to one another on SDS-PAGE autoradiograms at all times (Fig. 2). Hubbard and Cohn (9) obtained similar results with externally iodinated L cell PM proteins interiorized by phagocytosis. The fact that all PL polypeptides are digested in concert suggests that membrane degradation is a quantal process. For example, digestion may involve exposing whole segments of membrane to the lysosomal milieu, as in multivesicular bodies.

That the rapid and slow components of membrane turnover involve the same polypeptides suggests either that the cell recognizes portions of the membrane and marks these for rapid digestion or that a constant aliquot of membrane is randomly processed. If we consider a selective process to be more feasible, then what determines the nature of the signal? One possibility that has been invoked for other autophagic processes is that altered or denatured membrane components are responsible. These may either occur through natural pathways or, in this specific case, be the result of iodination. In either case, lysosomal acid hydrolases would find these denatured proteins more readily digestible substrates (3). Another possibility is that the lifetime of a PL membrane protein is related to its length of exposure to the acidic and hydrolase-rich environment of the lysosome. Rapidly digested membrane may be that immobilized in the lysosome, whereas membrane that exhibits a longer lifespan does so by virtue of leaving the PL for the cell surface, rather than any intrinsic insusceptibility.

The Centrifugal Flow of Vacuolar Membrane

The unique finding of this study is that membrane proteins introduced into the PL can escape degradation and return to the cell surface. Previous studies on the fate of PL membrane (9, 19) were not designed to detect this. We performed three types of experiments to demonstrate the extensive efflux of membrane components within 15–30 min after internal iodination of the cells. (*a*) EM-ARG indicated that the majority of the radiolabel originally on the PL membrane was now distributed on the PM. (*b*) The radiolabeled polypeptides became susceptible to extracellular proteolysis. (*c*) The PM could be isolated and shown to contain a spectrum of labeled proteins identical to those iodinated on the PL.

Our observations that return of all PL polypeptides to the PM is rapid and concerted suggests that the process occurs by a bulk movement of assembled membranes. We favor the idea that small membrane vesicles bud from the PL and subsequently fuse with the PM. These vesicles and their route of return have not been identified directly.

Several alternative explanations for the presence of radiolabel on the PM were considered. (*a*) Radiolabeled phagolysosome membrane was not being brought back to the surface,

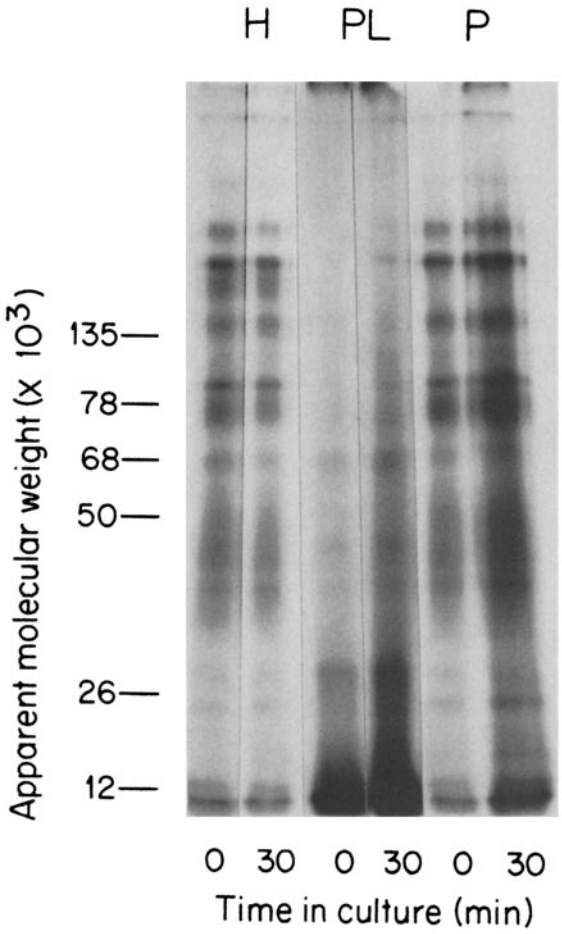


FIGURE 7 Movement of radioiodinated PM proteins into latex PL. After ingestion of PS-latex, macrophage surfaces were iodinated at 4°C, using soluble LPO. Cells were homogenized and fractionated immediately (*0*) or after return to culture for 30 min (*30*). Samples of the 10/25% interface fraction (*PL*) at both time-points, containing an equal amount of latex, were compared by SDS-PAGE. The homogenate (*H*) and pellet (*P*) lanes received approximately equal amounts of radiolabel (predominantly PM). The dried gel was exposed on Kodak XR-1 film with an enhancing screen.

because of exocytosis of LPO-latex. Sensitive light-scattering measurements of cell lysates and direct counts of particles in cells by phase microscopy showed that all of the latex originally ingested remained within the cell. (*b*) Monoiodotyrosine is not reused by the cell in protein synthesis (14). Were this occurring, the spectrum of radiolabeled proteins in the cell would shift with time to resemble the spectrum of the major cell proteins, but this did not occur (Fig. 2). (*c*) No new iodination occurred when cells were returned to culture (Table II). Therefore the radiolabeled polypeptides found on the PM moved there from the site of iodination in the PL.

Although membrane returns from PL to the cell surface, the contents of the lysosome matrix, including acid hydrolases and exogenous molecules, do not seem to follow such a pathway. Neither serum albumin (5) nor HRP (18) once internalized by the cell is found in the extracellular milieu. It is possible, however, that molecules that are tightly associated with the luminal surface of the PL maintain this contact during membrane flow. The mechanism by which the constituents of the lysosomal matrix are excluded is speculative. The size of the channel or pore into the forming vesicle may provide a barrier to macromolecules, much as does the pore-mediated barrier that presumably restricts transport of proteins in endothelial endocytic vacuoles and fenestrae (16). Alternatively, if the lysosome contents are in a viscous state, rapid vesiculation might remove membrane before large macromolecules (but not water and small ions) could equilibrate within the buds. Finally, the hydrostatic pressure within the forming vesicle may be larger than that in the remaining PL, preventing contents from entering.

Is membrane returned to all areas of the cell surface, or are there specific sites for insertion? By 15 min, autoradiographic grains appear randomly distributed on the plasma membrane. Measurements of the diffusion constants for membrane proteins in the plane of the bilayer are in the range of 5×10^{-9} to 10^{-11} cm^2/s (4, 10, 13). Only at the fastest rate would lateral diffusion of membrane proteins from a small number of specialized insertion sites produce the rapid randomization of radiolabel we observe; but, even then, these sites would have to be distributed evenly around the plasmalemma. We think it likely that membrane returns to multiple sites on both the apical and basal surfaces of cultured macrophages, after which diffusion could produce further randomization of radiolabeled proteins. Returning membrane may pass through the Golgi complex (6) or GERL (7). Perhaps by conducting EM-ARG studies at very early time-points we shall be able to determine whether membrane passes preferentially (or obligatorily) through a certain region of the cell.

Centripetal Membrane Flow

The existence of rapid membrane movement from PL to PM implies that there must be a new source of membrane to maintain the size of the PL compartment. Our previous stereological analysis suggested that the major source of this membrane could be newly derived PV (17). Additional results derived by the different techniques employed in the present study also support this idea. (*a*) Thorotrast is delivered to all LPO-latex PL (Fig. 5), indicating that all are undergoing fusion with PM-derived PV. (*b*) HRP accumulates in PL at a constant rate for at least 2 h (Fig. 6). (*c*) After iodination of the cell surface, cultured cells internalize a representative sample of PM into the PL (Fig. 7). The current studies of Mellman et al. (in preparation) using LPO-catalyzed iodination within PV indicate directly that PV membrane is equivalent in composition to PM. Thus, there appears to be a continuous movement of PM into PL via PV that we think largely balances the flow of membrane from PL to PM.

Membrane Recycling during Endocytosis

The data presented in this and the accompanying paper establish that PM polypeptides and, presumably, segments of fully assembled PM flow from the cell surface into the vacuolar apparatus and return to the cell surface. This recycling of membrane is both rapid and extensive, occurring within minutes, and is consistent with the extensive membrane internalization first appreciated during a stereological analysis of the pinocytic process (17). In both macrophages and L cells, large segments of their surface area, 186% and 48% respectively, were internalized each hour as PV without an appreciable alteration in the surface area or volume of the total cell or its vacuolar apparatus. It is plausible, therefore, that the efflux of membrane to the cell surface demonstrated in this study is tightly coupled to and balanced by the rate of pinocytosis.

Stereological approaches employed in the past were not able

to characterize definitively the centrifugal flow of membrane nor its composition. For this purpose, latex PL selectively labeled by an enzymatic technique served as the focus for more detailed analysis. Movement of radiolabeled membrane proteins from PL to PM was demonstrated, as well as the influx of a similar group of polypeptides from PM to PL. We would assume that movement of PM into and out of the latex PL is a constitutive process, even though we only followed some aspects of it, e.g., the movement of radiolabel from PL to PM for 30 min. For example, PV deliver HRP to latex PL at a constant rate for at least 2 h; yet the latex PL, like other secondary lysosomes (17), never swells with excess membrane, so the PM that joins PL via PV most likely returns to the surface as well.

Other investigators have obtained evidence consistent with membrane recycling in mammalian cells, although their approaches were more indirect. Farquhar (6) tagged the surface of rat anterior pituitary cells with cationized ferritin and demonstrated that electron-dense marker was pinocytosed and subsequently found in lysosomes, Golgi complex, and condensing granules. Assuming that the movement of cationized ferritin was tracing the natural movement of PM into secretory granules, endocytosed PM could then be recycled for use in subsequent secretory events. Schneider et al. (15) reported that fibroblasts that had internalized radiolabeled goat anti-rabbit IgG released some of this radiolabel when rabbit IgG was added to cultures. They postulated that the rabbit IgG bound to the PM, was interiorized by pinocytosis, complexed with the goat anti-rabbit IgG within the lysosomes, and shuttled it back to the cell surface where it was released into the culture medium.

Consequences of Membrane Recycling

The vacuolar apparatus has traditionally been viewed as a system of structurally unique and well-defined compartments. The route to the lysosome was considered a unidirectional path taken by exogenous molecules and membrane destined for digestion. The concept of the vacuolar system that emerges from this study is one of a dynamic system of membranes that interact continuously and extensively with the plasmalemma. In this view, the potential role of the vacuolar apparatus is expanded from that of a digestive system to an intimate part of the PM capable of influencing several aspects of PM physiology. Some examples are of considerable current interest and will be summarized.

Membrane recycling allows for the efficient delivery of receptor-bound ligands to the lysosomes without obligatory loss or degradation of the receptor itself. Anderson et al. (1) have proposed that recycling of fibroblast low-density lipoprotein (LDL) receptors accounts for the extensive and continued uptake of LDL in the absence of receptor synthesis. In contrast, interaction of other ligands, such as epidermal growth factor, with their receptors leads to removal of the receptors from the cell surface (2), or so-called down regulation. It is possible that a consequence of ligand binding is to block recycling of membrane, trapping the complex in the lysosome. We favor the idea that some feature of the receptor or complex rather than the ligand may determine whether the complex is trapped in the lysosome (down regulation) or whether the ligand is discharged in the lysosome, followed by recycling of the free receptor.

Vesicular membrane flow may be used by the cell to deliver membrane from one area to another. Although recycling appeared to be a random process under the culture conditions we studied, it is possible that large areas of membrane could be directed via the vacuolar system to regions of the cell where they are needed during cell movement or spreading, e.g., when macrophages respond to a chemoattractant. We are currently investigating this possibility.

Rapid membrane flow and recycling may be involved in membrane turnover. Our data show that interiorized PM may fuse with lysosomes but escape degradation. This may occur because degradation of normal membrane may be a random event so that very little is degraded on any one trip through the lysosome. However, altered membrane may be specifically recognized and rapidly degraded by the lysosome. Because large amounts of PM continuously flow into and out of the vacuolar system, this mechanism would allow the cell to rapidly and continuously survey the integrity of the PM.

Finally, there is the suggestion that the vacuolar system could serve as an intermediate in the renewal and synthesis of PM. This is based on the information that the polypeptide compositions of the PM and PL are virtually indistinguishable (12). The input of new membrane into the vacuolar apparatus presumably comes from the membrane of the primary lysosome, which in turn originates in the Golgi or GERL area of the cell. If typical PM surrounds the primary lysosome, this would imply that new segments of PM are first inserted into the secondary lysosome and then reach the cell surface during the recycling process.

We gratefully acknowledge the expert technical assistance of Richard Huntley and Judy Adams.

This work was performed while W. A. Muller held a predoctoral traineeship awarded under National Institute of General Medical Sciences grant no. 5 T05 GM02243. R. M. Steinman is an Irma T. Hirschl Fellow of The Rockefeller University; Z. A. Cohn is the recipient of U. S. Public Health Service grant no. AI07012.

Received for publication 7 December 1979, and in revised form 22 February 1980.

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